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The overall objective of this project is to develop an assay to detect small numbers of breast cancer cells in a patient's peripheral blood. This assay would be used to test the hypothesis that the presence of circulating breast cancer cells would be predictive of stage at presentation or relapse. The first stage of this project has been to optimize a quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) to quantify breast cancer-specific RNA molecules in the peripheral blood. The result of this initial work was to select keratin 19 (K19) RNA as the best target for detecting small numbers of breast cancer cells. We have also selected an internal reference RNA (β_2-microglobulin), and we have optimized our protocol for extracting RNA from patient blood samples. Having optimized these parameters, we plan to use the qRT-PCR assay for K19 RNA to study patients with breast cancer, both at the time of presentation and after treatment.

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INTRODUCTION

The overall objective of this project is to develop an assay to detect small numbers of breast cancer cells in a patient's peripheral blood. This assay would be used to test the hypothesis that the presence of circulating breast cancer cells would be predictive of stage at presentation or relapse. The first stage of this project has been to optimize a quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) to quantify breast cancer-specific RNA molecules in the peripheral blood. The result of this initial work was to select keratin 19 (K19) RNA as the best target for detecting small numbers of breast cancer cells. We have also selected an internal reference RNA (β_2-microglobulin), and we have optimized our protocol for extracting RNA from patient blood samples. Having optimized these parameters, we plan to use the qRT-PCR assay for K19 RNA to study patients with breast cancer, both at the time of presentation and after treatment.

WORK ACCOMPLISHED

Task 1: To develop molecular tests to quantify breast cancer cells in the peripheral blood

During the past year, we have devoted our efforts to optimizing the qRT-PCR assay to detect small numbers of circulating breast cancer cells. To develop such an assay one needs to design PCR primers and probes, identify a cell line containing the RNA targets, optimize PCR conditions, evaluate the PCR method for its ability to quantify the target RNA, select an internal reference RNA, optimize methodology for purifying RNA from peripheral blood, and use the optimized assay to quantify the RNA target in normal peripheral blood to establish a reference range.

Primer and probe design

Taqman primers and probes were designed to detect K19 and MUC1 using PrimerExpress 1.0 software (Applied Biosystems). K19 was selected since it is expressed in neoplastic epithelial cells (including breast cancer cells) and not in blood cells. MUC1 was selected since it is known to be expressed in breast cancer, and since the MUC1 protein is used as a serologic tumor marker to determine patients' tumor burden. Primers and probes were also designed for three RNAs that were to be tested as internal reference RNAs. The prospective reference RNAs were ribosomal RNA rRNA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β2-microglobulin (β2m). All primers and probes were designed to span an intron, which would prevent unwanted detection of DNA. In addition, since it is known that there are a number of pseudogenes for K19 (1), K19 primers were designed to detect only the "genuine" K19 RNA and not the pseudogenes. The primers and probes are listed in Table 1.

Identification of a cell line expressing breast cancer RNAs

Since a significant amount of breast cancer RNA would be required to optimize the qRT-PCR assay, we needed a source of breast cancer RNA that could be maintained in the laboratory. Thus, we evaluated two breast cancer cell lines for the presence of K19 and MUC1 RNA. The cell lines used were SKBR3 and BT20. Both cell lines were found to contain ample K19 and MUC1 RNA, so the SKBR3 line was selected for further study since it was the easiest to maintain in tissue culture.

Table 1. Taqman primers and probes

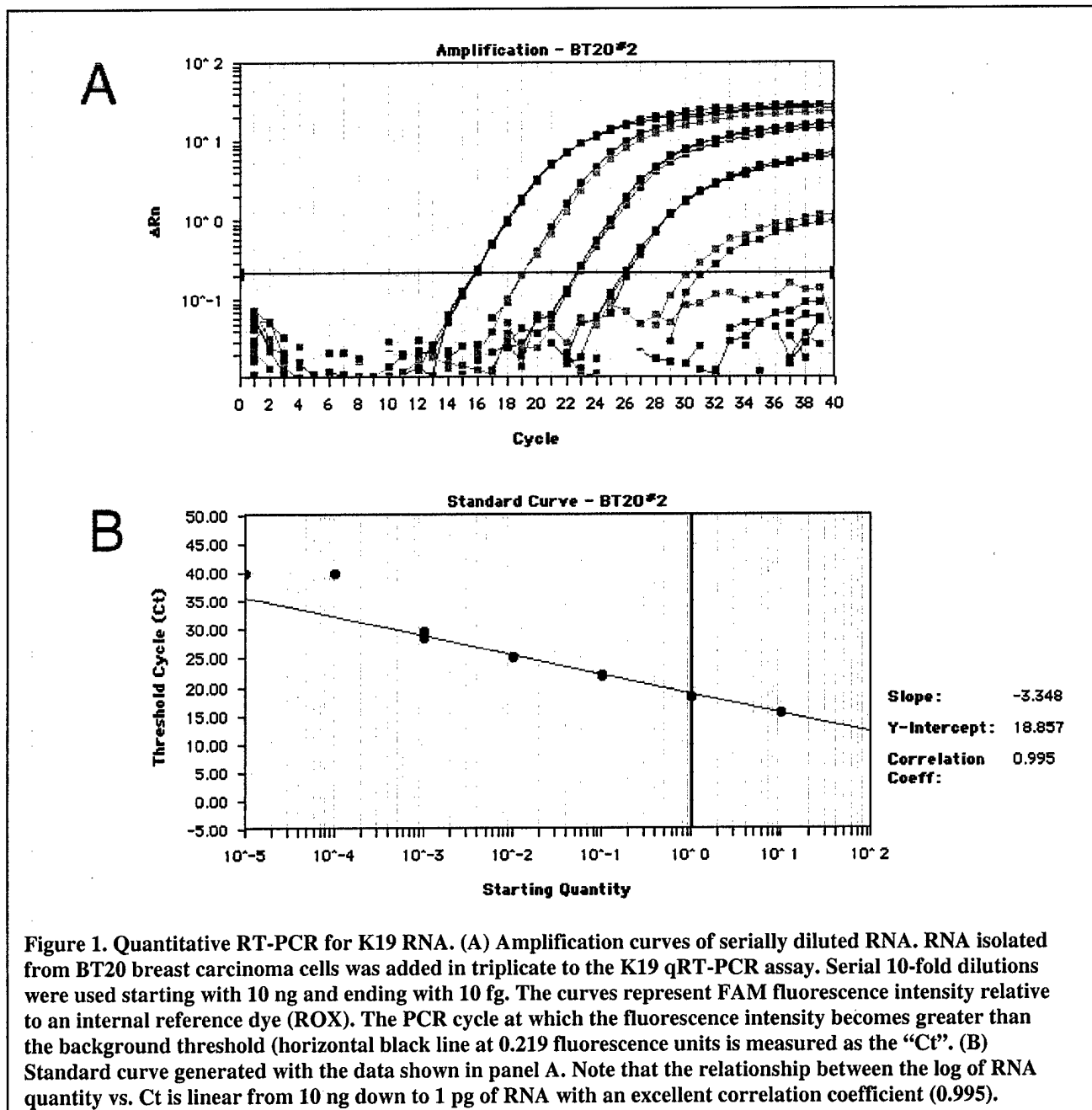| RNA Target	Forward Primer	Reverse Primer	Probe
K19	GCGGCGCACC CTTCA	GTTTCTGCCA GTGTGTCTTC CA	CAGTCACAGCTGAGCATGA AAGCTGCC
MUC1	TGCGCTGGCC ATTGTCT	AGCTGCCCGT AGTTCTTTCG	TCTCATTGCCTTGGCTGTCT GTCAGTG
rRNA	CGGCTACCAC ATCCAAGGAA	GCTGGAATTA CCGCGGCT	TGCTGGCACCAGACTTGCC CTC
GAPDH	CCACATCGCT CAGACACCAT	CCAGGCGCCC AATACG	AAGGTGAAGGTCGGAGTCA ACGGATTTG
β2-microglobulin	TGCTCGCGCT ACTCTCTCTTT	GGATGACGTG AGTAAACCTG AATCT	CCTGGAGGGCATCCAGCGT ACTCC

Optimization of qRT-PCR

For each target RNA, a series of PCR reactions was performed in which the concentrations of the forward and reverse primers were varied from 100 nM to 900 nM. The probe concentration was held constant at 100 nM. The maximum amount of PCR product formed for the targets tested was obtained when the primer concentrations were 900 nM for both the forward and reverse primers. By using these primer concentrations and varying the probe concentration, we determined that the optimum probe concentration was 100 nM for all primer/probe sets.

Selection of RNA target for qRT-PCR

We initially tried K19 and MUC1 as targets for quantification using RNA isolated from SKBR3 and BT20 cells. We found that K19 was approximately 100-fold more sensitive a marker for breast cancer than MUC1, most likely due to its higher abundance in breast cancer cells.

Having established the PCR conditions, we performed qRT-PCR on RNA isolated from BT20 cells. Serial 10-fold dilutions of total RNA were prepared, and qRT-PCR was performed using 10 ng down to 10 fg of total RNA. The results of this experiment are shown in Figure 1. Figure 1A shows amplification curves using K19 primers and probes. As the PCR products accumulate, the fluorogenic probe anneals to the PCR products. Taq polymerase, due to its 5'→3' exonuclease activity (2), digests probe that is annealed to the PCR products. The probe contains a fluorochrome (FAM) at the 5' end and a fluorescent quencher (TAMRA) on the 3' end. The intact probe generates very little fluorescent signal, but when the probe is digested by Taq polymerase, FAM is spatially separated from TAMRA, resulting in an increase in the fluorescent signal. Thus as the PCR products accumulate, fluorescence increases as shown in Figure 1A. The cycle at which the fluorescence signal increases above a given threshold (the horizontal line in Figure 1A is inversely proportional to the starting amount of RNA. This cycle is referred to as the "threshold cycle", or Ct. Figure 1B shows that there is a linear relationship between Ct and the log of the starting RNA quantity. The assay is linear from 10 ng down to 1 pg of total RNA. 1 pg of RNA corresponds to about 1 breast cancer cell in 10^6 non-breast cancer cells. When a similar experiment was performed using MUC1 as a target, the assay could only detect a MUC1 signal in 100 pg of RNA. Thus the sensitivity of the MUC1 assay is 2 logs lower than that of the

Figure 1. Quantitative RT-PCR for K19 RNA. (A) Amplification curves of serially diluted RNA. RNA isolated from BT20 breast carcinoma cells was added in triplicate to the K19 qRT-PCR assay. Serial 10-fold dilutions were used starting with 10 ng and ending with 10 fg. The curves represent FAM fluorescence intensity relative to an internal reference dye (ROX). The PCR cycle at which the fluorescence intensity becomes greater than the background threshold (horizontal black line at 0.219 fluorescence units is measured as the "Ct". (B) Standard curve generated with the data shown in panel A. Note that the relationship between the log of RNA quantity vs. Ct is linear from 10 ng down to 1 pg of RNA with an excellent correlation coefficient (0.995).

K19 assay. Therefore, we have focused our efforts on optimizing the K19 assay for use in this study.

Selection of an internal reference RNA

An internal reference RNA is used as a normalization control to correct for variations in RNA loading and other factors that might affect PCR efficiency for a given RNA sample. An ideal reference RNA is one that is present in all cells at constant levels, is comparable in quantity to the test RNA (i.e., K19), and for which primers and probes can be designed easily. We evaluated rRNA, GAPDH, and β2m RNAs as possible internal reference RNAs. rRNA was present at too high a level for accurate quantification. GAPDH is also expressed at a high level.

In addition, there are multiple pseudogenes for GAPDH in the genome (3, 4), making it difficult to design primers and probes. Finally, $\beta 2m$ has been used successfully as an internal reference RNA for a RT-PCR for BCR-ABL currently in use in our laboratory (5), so we have experience with this gene. When used as a target for qRT-PCR, $\beta 2m$ gave reproducible amplification. For these reasons, we have selected $\beta 2m$ as our internal reference RNA.

We investigated whether we could simultaneously amplify K19 and b2m in a single multiplex PCR reaction, but the sensitivity to detect K19 RNA decreased by 100-fold, so we decided to perform the two PCR reactions separately.

Optimization of RNA preparation

To test the clinical sensitivity of the qRT-PCR assay for K19 RNA, we added known numbers of SKBR3 cells to normal peripheral blood samples. The blood samples were made to contain from 1 in 100 to 1 in 10^6 SKBR3 cells per white blood cell. RNA was prepared from the spiked specimens and subjected to qRT-PCR.

RNA was initially prepared from whole blood, but this resulted in the ability to detect only 1 SKBR3 cell in 100 white cells. This level of sensitivity is unacceptably low for detecting minimal numbers of breast cancer cells in the peripheral blood. We investigated whether the RNA preparation method would affect sensitivity. Purifying RNA using a phenol-based method (Trizol) or using RNA preparation kits from Qiagen and Gentra Systems resulted in similarly low sensitivity. Furthermore, RNA prepared using the Gentra Systems kit resulted in significant levels of DNA contamination. Some DNA contamination was observed with the Qiagen kit as well. To determine if DNA contamination affected assay sensitivity, a DNase step was added. This resulted in less DNA contamination, but this step did not improve the sensitivity of the assay, so it was not used in subsequent experiments.

We considered the possibility that red blood cell contaminants might be adversely affecting the sensitivity of the K19 RT-PCR assay, so we added a Ficoll gradient density centrifugation step to remove red cells and consequently enrich for white cells (and presumably cancer cells as well). We tried two Ficoll methods, 1) Vacutainer tubes (Becton-Dickinson) containing a Ficoll preparation and a solid-phase cell separator layer, and 2) standard Ficoll gradients made in the laboratory. The Vacutainer tubes were found to produce false-positive results, i.e., K19 signals were obtained from normal peripheral blood that had not been spiked with tumor cells. In contrast, using Ficoll gradients made in the laboratory, we were able to get cleanly negative unspiked controls, and we were able to detect as few as 5 SKBR3 cells in 10^7 white cells. This level of sensitivity approaches the theoretical limit of PCR sensitivity, and thus will be acceptable for a clinical assay.

Finally we have entered into collaboration with Dr. Nancy Kiviat at the University of Washington. Dr. Kiviat is interested in using cytological techniques to detect minimal residual disease in breast and prostate cancer. Dr. Kiviat's laboratory uses a magnetic bead technique to enrich for epithelial cells in blood samples. We will be investigating whether such an enrichment technique can improve the sensitivity of our qRT-PCR assay.

Establishment of reference range

We have just completed method optimization and will be beginning testing normal subjects shortly. We have performed a pilot study to determine whether we need to be concerned about contamination of blood samples with epidermal cells. It is theoretically possible that the

presence of skin cells could yield false positive results. To make this determination we collected two tubes of blood from 10 normal subjects and performed qRT-PCR for K19 RNA on both tubes. In no case did we detect K19 RNA in the first or second tube, indicating that contamination with skin cells is not likely to be a significant analytical problem.

Task 2: To apply the test to untreated breast cancer patients

Since the assay has just recently been optimized, we have not yet begun collecting specimens from breast cancer patients. We plan to collect specimens from the University of Washington Medical Center (supervised by Dr. Melanie Palomares) and from the Harborview Medical Center (supervised by Dr. Diana Sabath). In addition, we will receive additional patient specimens through our collaboration with Dr. Kiviat. Dr. Kiviat has an ongoing research program in Senegal and will be collecting specimens from patients with primary diagnoses of breast cancer in Africa and returning the specimens to Seattle.

We will be collecting peripheral blood samples from patients with new breast cancer diagnoses with all clinical stages of disease. We would like to get as many samples as possible from patients with Stage III and IV disease, since we expect these to provide more positive results. This will be the primary focus of our work in the coming year.

Task 3: To apply the test to treated breast cancer patients to determine if the test can predict disease progression

Since we are just beginning to collect specimens from first-time patients, we have not yet begun work on this task.

Key Research Accomplishments

- Selection of a breast cancer-specific RNA (K19) and optimization of qRT-PCR reaction
- Selection of an internal reference RNA (b2m) and optimization of qRT-PCR reaction
- Optimization of protocol to isolate RNA from peripheral blood
- Establishment of working relationship with oncology clinics in Seattle and Senegal from which to get patient specimens

Reportable Outcomes

Abstracts

Palomares, M. R., Gralow, J. R., Koehler, K. M., Sabath, D. E. (2000) The Detection of Circulating Breast Cancer Cells Using Quantitative Cytokeratin-19 mRNA Real-Time PCR. Submitted to the Year 2000 Smith-Kline National Medical Oncology Fellows' Forum.

Palomares, M. R., Gralow, J. R., Koehler, K. M., and Sabath, D. E. (2000) Detection of Circulating Breast Cancer Cells Using Quantitative Cytokeratin-19 mRNA Real-Time PCR. Submitted to the MD Anderson Cancer Center Medical Oncology of Breast Cancer Fellows Program.

Presentations

Palomares, M. R., Gralow, J. R., Koehler, K. M., Sabath, D. E. "The Detection of Circulating Breast Cancer Cells Using Quantitative Cytokeratin-19 mRNA Real-Time PCR," Year 2000 Smith-Kline National Medical Oncology Fellows' Forum, poster presentation on March 3, 2000.

Palomares, M. R., Gralow, J. R., Koehler, K. M., Sabath, D. E. "The Detection of Circulating Breast Cancer Cells Using Quantitative Cytokeratin-19 mRNA Real-Time PCR," MD Anderson Cancer Center Medical Oncology of Breast Cancer Fellows Program, platform presentation on July 29, 2000.

Palomares, M. R. "Minimally Invasive Biomarkers for Breast Cancer Prevention and Early Detection," Fred Hutchinson Cancer Research Center Clinical Research Division Seminar, Aug 14, 2000.

Palomares, M. R. "Minimally Invasive Biomarkers for Breast Cancer Prevention and Early Detection," Harbor-UCLA Oncology Grand Rounds, Sept 15, 2000.

Palomares, M. R. "Minimally Invasive Biomarkers for Breast Cancer Prevention and Early Detection," City of Hope Center for Cancer Genetics Technology Transfer Research Seminar, Sept 19, 2000.

Training supported by this award

Dr. Melanie Palomares, a Senior Fellow in Oncology, conducted this research project in partial fulfillment of her requirements for board eligibility in Hematology-Oncology.

Conclusions

The work accomplished so far has been to validate an assay to quantitate K19 RNA in the blood of breast cancer patients, which should reflect the number of circulating breast cancer cells. Although we have not yet begun clinical trials with this assay, we have demonstrated that we should be able to detect 1 breast cancer cell in 10^6 white blood cells. In the next phase of our study, we will determine whether this assay has clinical utility for both determining a patient's prognosis at initial presentation and predicting the likelihood of progressive disease on follow-up. We will also be evaluating whether using magnetic bead enrichment of epithelial cells will improve the assay sensitivity. If our efforts are successful, this assay may come to be used in the routine care of cancer patients. We hope that by detecting cancer cells in the blood, patients may be treated earlier for recurrent/metastatic disease and that this will translate into improved patient survival.

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Abstract Type: (check one)
X Research, Clinical   ☐ Research, Basic Science
☐ Clinical Case Study

Abstract Title: (bold, initial capitals) **The Detection of Circulating Breast Cancer Cells Using Quantitative Cytokeratin-19 mRNA Real-Time PCR**

Authors: (underline presenter) Melanie R. Palomares, M.D., Julie R. Gralow, M.D., Karen M. Koehler, and Daniel E. Sabath, M.D., Ph.D., University of Washington, Division of Medical Oncology and Department of Laboratory Medicine, Seattle WA

Abstract Text

The development of a quantitative method of detecting circulating breast cancer cells would be useful in following breast cancer patients for response to treatment and early detection of recurrence. To accomplish this, we developed a quantitative real-time RT-PCR assay using cytokeratin-19 mRNA.

RNA was extracted from peripheral blood samples collected from women 30 years of age or older who were seen at the UW Cancer Center. Real time PCR was done on an ABI 7700 (Perkin-Elmer), using RNA extracted from SKBR3 cells as a standard. A standard curve was constructed so that results could be quantitatively expressed as SKBR3 RNA equivalents.

A total of 37 blood samples were collected, and were analyzed as follows: Group 1 consisted of women with non-breast cancers (2 lung, 2 colon, 1 head and neck, 1 gynecologic, and 5 hematologic), Group 2 included breast cancer patients with no evidence of active disease, and Group 3 consisted of breast cancer patients currently receiving treatment for metastatic disease. Results are shown below, along with p-values for two-sample t-tests comparing Groups 2 and 3 to Group 1:

	N	Mean RNA level (ng)	Standard Deviation	p-value
Group 1	11	0.0293	0.0615	-
Group 2	16	0.0968	0.2955	0.46504
Group 3	10	0.2610	0.4048	0.10547

Progressively increasing mean RNA equivalents were observed to correspond to increasing breast cancer tumor burden, however differences between groups were not statistically significant. Low sample size and high variability within Groups 2 and 3 probably account for this. We are currently recruiting more patients, incorporating an internal assay control to decrease variability, and considering epithelial enrichment of blood samples to enhance sensitivity. We also plan to analyze longitudinal samples with clinical correlation.

Abstract submitted to the MD Anderson Cancer Center Medical Oncology of Breast Cancer Fellows Program, July 2000

Detection of Circulating Breast Cancer Cells
Using Quantitative Cytokeratin-19 mRNA Real-Time PCR

Melanie R. Palomares, M.D., Julie R. Gralow, M.D., Karen M. Koehler, and Daniel E. Sabath, M.D., Ph.D., University of Washington, Division of Medical Oncology and Department of Laboratory Medicine, Seattle WA.

The development of a quantitative method for detecting circulating breast cancer cells would be useful in following breast cancer patients for response to treatment and early detection of recurrence. To accomplish this, we developed a quantitative real-time RT-PCR assay using cytokeratin-19 mRNA as a marker for breast cancer cells.

RNA was extracted from peripheral blood samples collected from women 30 years of age or older who were seen at the UW Cancer Center. Real time PCR was done on an ABI 7700 (Perkin-Elmer), using RNA extracted from SKBR3 cells as a standard. A standard curve was constructed so that results could be quantitatively expressed as SKBR3 RNA equivalents.

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Progressively increasing mean RNA equivalents were observed to correspond to increasing breast cancer tumor burden. ROC analysis revealed that using a cut-off RNA level of 0.03ng maximized the sensitivity and specificity of our assay to 60% and 82%, respectively. Logistic regression analysis revealed that women with metastatic breast cancer were 6.6 times more likely to have an RNA level >0.03ng than all other women (p=.02). However, this odds ratio decreased to 5.0 when adjusted for date of PCR run (p=.06).

We are currently incorporating an internal assay control to decrease interassay variability, and we are considering epithelial enrichment of blood samples to enhance sensitivity. We also plan to recruit more patients to increase power, and to analyze longitudinal samples for clinical correlation.